

*REMARKS**The Present Invention*

The present invention is directed to isolated cancer peptides or functionally equivalent variants thereof, compositions thereof, and immunogens comprising the compositions.

The Pending Claims

Claims 3, 5-8, 10, 12-15, 26, 28, 29, 67-77, 83-85, and 87-103 are pending, of which claims 3, 5-8, 10, 12-15, 67-77, and 87-97 are directed to isolated cancer peptides and variants thereof, claims 26 and 98 are directed to compositions, and claims 28, 29, 83-85, and 99-103 are directed to immunogens. Claims 89-91 are indicated to be objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

The Amendments to the Claims

Claims 3 and 26 have been amended to delete the phrase "about 10 contiguous amino acids of SEQ ID NO: 4." The phrase "is immunologically recognized by antigen specific cytotoxic T lymphocytes" has been changed to "stimulates cancer antigen specific T lymphocytes" which is supported by the specification at, for instance, page 9, lines 23-26. The phrase "at least 85% sequence homology" has also been deleted. Claims 3 and 26 now recite "wherein the functionally equivalent variant has at least 85% sequence identity with amino acids 53-62 of SEQ ID NO: 4" which is implicitly supported by the specification at, for example, Table 7, which lists several examples of peptides that are at least 85% identical in sequence to amino acids 53-62 of SEQ ID NO: 4. No new matter has been added by way of these amendments. Furthermore, the amendments made herein do not raise new issues that would require further consideration and/or search.

Discussion of the Indefiniteness Rejection

The Office Action maintains the rejection of claims 3, 5-8, 10, 12-15, 26, 67-77, and 87 under 35 USC 112, second paragraph, as allegedly indefinite. Specifically, the Office Action contends that the term "functionally equivalent variant" is unclear, since the function is not clearly defined by the claims. The Office argues that the rejected claims are unclear because the limitation of being "recognized by antigen specific cytotoxic T lymphocytes" is not an actual function. Rather, it is a property or characteristic of a "functionally equivalent variant." Claims 3 and 26 have been amended to recite "wherein said peptide or functionally equivalent variant stimulates cancer antigen specific T lymphocytes" which is supported by

the specification at, for example, page 9, lines 23-26, and Tables 6 and 7, both of which teach peptides stimulating cancer antigen T lymphocytes, as evidenced by the release of GM-CSF by T lymphocytes. The amended claims are clear as to the function of the claimed peptides or variants thereof. Therefore, in view of the amendments to the claims, this rejection is moot.

Discussion of the Written Description Rejection

The Office Action maintains the rejection of claims 3, 5-8, 26, 67, 68, and 87 under 35 USC 112, first paragraph, as allegedly lacking a written description. In particular, the Office contends that there is inadequate written description for the homologous cancer peptides from other mammalian sources that are encompassed by the broad claims. The rejection is traversed for the reasons set forth below.

Claims 3 and 26 have been amended to delete the term "sequence homology" and to instead recite "wherein the functionally equivalent variant has at least 85% sequence identity with amino acids 53-62 of SEQ ID NO: 4" which amendment is implicitly supported by the specification at, for instance, Table 7. Specifically, the functionally equivalent variants found in Table 7 (e.g., SEQ ID NOs: 34-38, 41, and 42) have at least 85% sequence identity with amino acids 53-62 of SEQ ID NO: 4 and, as evidenced by the release of GM-CSF by T lymphocytes, such functionally equivalent variants stimulate cancer antigen specific T lymphocytes. Accordingly, the originally-filed specification adequately describes the peptides and variants thereof of the amended claims. Applicants, therefore, request that the written description rejection be withdrawn.

Discussion of the Written Description Rejection – New Matter

The Office Action maintains the rejection of claims 3, 5-8, 10, 12, 26, 67-69, 72, 87, 88, and 92-103 under 35 USC 112, first paragraph, as allegedly lacking written description. More specifically, the Office Action contends that the rejected claims contain new matter, since the originally-filed specification allegedly does not adequately describe "about ten contiguous amino acids of SEQ ID NO: 4 that include amino acids 55-62 of SEQ ID NO: 4 or amino acids 127-136 of SEQ ID NO: 4" or a functionally equivalent variant thereof. The Office Action also questions where "at page 9 is it contemplated that the cancer peptide 'portions thereof' includes amino acids 55-62 of SEQ ID NO: 4 or includes amino acids 127-136 of SEQ ID NO: 4 and is about 10 amino acids in length as well as peptides having at least 85% sequence homology to said portions?" This rejection is traversed for the reasons set forth below.

As framed by the court in *In re Rasmussen*, 650 F.2d 1212, 211 USPQ 323 (CCPA 1981), the concept of new matter is properly employed as a basis for objection to amendments to the abstract, specification or drawings attempting to add new disclosure to that originally presented. See also the Manual of Patent Examining Procedure (MPEP) 2163.01. In the instant case, support for each limitation of the amended claims can be found in the originally-filed disclosure. The term "isolated" is supported by the specification at, for example, page 13, lines 15-18. The term "cancer peptide" is supported by the specification at, for instance, page 9, lines 2-3. Although claims 3 and 26 have been amended to delete it, the phrase "about 10 contiguous amino acids of SEQ ID NO: 4" is supported by the specification at, for instance, page 8, line 28-page 9, line 1. The limitations "amino acids 55-62 of SEQ ID NO: 4" and "amino acids 127-136 of SEQ ID NO: 4" are supported by the specification at, for example, Tables 6 and 7, Figures 3A-1 and 3A-2. The term "functionally equivalent variants" is supported by the specification at, for instance, page 13, lines 10-14. The phrase "at least 85% sequence identity with amino acids 53-62 of SEQ ID NO: 4" is implicitly supported by Table 7. The phrase "optionally 1 to about 10 additional contiguous amino acids of SEQ ID NO: 4 at the N-terminus of the cancer peptide" is supported by the specification at, for example, page 11, lines 3-5. The phrase "stimulates cancer antigen specific T lymphocytes" is supported by the specification at, for instance, page 9, lines 25 and 26. The term "composition" is supported by the specification at, for instance, page 13, line 27-page 14, line 1. Therefore, the subject matter of the instant claims is not new matter.

It appears that the Office requires that the literal language of the claims be *explicitly* supported by the originally-filed specification. This is not required. Rather, to comply with the written description requirement of 35 USC 112, paragraph 1, each claim limitation must be expressly, *implicitly*, or *inherently* supported by the originally filed disclosure. See, MPEP 2163.05 (a) (emphasis added).

For the reasons set forth above, both the explicit and implicit teachings of the instant specification adequately support the pending claims, such that the written description is met. Applicants, therefore, request that the written description rejection be withdrawn.

Furthermore, possession of the instantly claimed invention is demonstrated by teachings of the specification at, for instance, page 9, lines 10-21, in combination with Tables 6 and 7. The specification at page 9, lines 10-21 states: "In one embodiment, the cancer peptide of the present invention comprises the amino acid sequence...(SEQ ID NO: 4) and cancer epitopes, *fragments*, or *derivatives* thereof." (emphasis added).

Tables 6 and 7 disclose several peptides consisting of (a) amino acids 55-62 of SEQ ID NO: 4 or amino acids 127-136 of SEQ ID NO: 4 and (b) optionally 1 to about 10

additional contiguous amino acids of SEQ ID NO: 4 at the N terminus of the cancer peptide. SEQ ID NOs: 25-30 are *all* specific examples of such peptides.

Table 7 also discloses functionally equivalent variant peptides that have at least 85% sequence identity with amino acids 53-62 of SEQ ID NO: 4. Specific examples of such variant peptides include SEQ ID NOs: 34-38, 41, and 42. *All* of these exemplary peptides stimulate cancer antigen specific T lymphocytes, as evidenced by the release of GM-CSF by T lymphocytes.

Accordingly, the originally-filed specification does, in fact, reasonably convey to one of ordinary skill in the art that the inventors, at the time the application was filed, had possession of the claimed invention. On this basis alone, the rejection should be withdrawn.

The Office contends that the specification on page 9, which generically describes portions of SEQ ID NO: 4, does not adequately support the narrower limitations of the present claims. The Office reiterates that a generic or a subgeneric disclosure cannot support a species unless the species is specifically described.

However, as stated in the previous Response to Office Action, the specification at, for example, Tables 6 and 7 provides a multitude of exemplary peptides within the scope of the claims. Therefore, in view of the multitude of *specifically described* exemplary peptides, the specification contains more than a generic disclosure, and adequately supports the present claims.

The Office further contends that the scope of peptide variants is too broad, stating that Tables 6 and 7 support only those peptides disclosed therein. However, the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species. See MPEP 2163.05. In the instant case, the originally-filed application (e.g., Tables 6 and 7) discloses sufficient description of several species of variants, such that a representative number of species is disclosed.

The Office Action also alleges that there is insufficient support for "about 10 contiguous amino acids of SEQ ID NO: 4 and optionally 1 to about 10 or 1 to about 5 additional contiguous amino acids of SEQ ID NO: 4." Claims 3 and 26 have been amended to delete "about 10 contiguous amino acids of SEQ ID NO: 4." Thus, the rejection as it pertains to this phrase is moot. The specification at, for instance, page 8, line 28, through page 9, line 1, states "In another embodiment, the tumor or cancer antigen is about 10 amino acids in length," and page 11, lines 3-5, states "Peptides having about 1 to about 10, preferably about 1 to 5 additional amino acids at the N-terminus...also form part of the present invention." Thus, the claimed subject matter is adequately supported by the originally-filed specification.

The Office further alleges that there is insufficient written support for a cancer peptide consisting of amino acids 43-62 of SEQ ID NO: 4 (SEQ ID NO: 45) in Tables 6 and 7. Although this peptide is not disclosed in Tables 6 and 7, it is disclosed on page 11, line 10, of the originally-filed specification. Accordingly, there is sufficient written support for this peptide.

The Office also contends that peptides having at least 85% sequence homology with amino acids 127-136 of SEQ ID NO: 4 and optionally 1 to about 10 or 1 to about 5 additional contiguous amino acids of SEQ ID NO: 4 are not disclosed. Claims 3 and 26 have been amended to exclude functionally equivalent variants amino acids 127-136 of SEQ ID NO: 4. The deletion of such subject matter is not to be construed as abandonment of the invention. Applicants reserve the right to pursue the deleted subject matter in another application.

The Office further alleges that Table 7 does not provide adequate written support for the broad limitation of just any amino acid substitution at amino acid 54 of the peptide consisting of amino acids 53-62 of SEQ ID NO: 4 (part (xi) of claim 88) or just any additional amino acid at the N-terminus of the cancer peptide consisting of amino acids 54-62 of SEQ ID NO: 4 (part (xii) of claim 88).

However, as stated above, the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species. In the instant case, the originally-filed application discloses sufficient description of several species of peptides having an amino acid substitution at position 54 and consisting of amino acids 53-62 (see, e.g., SEQ ID NOs: 34-38 of Table 7). Likewise, the originally-filed application discloses sufficient description of many species of peptides having additional amino acids of SEQ ID NO: 4 at the N-terminus of the cancer peptide consisting of amino acids 54-62 of SEQ ID NO: 4 (see, e.g., SEQ ID NOs: 26-30). Accordingly, a representative number of species of the claimed genus is disclosed, such that the written description requirement for the claimed genus is satisfied.

In view of the foregoing, all of the claim limitations are expressly, implicitly, or inherently supported by the originally filed disclosure. Thus, the pending claims accord with the written description requirement, and Applicants request that the written description rejection for alleged new matter be withdrawn.

Discussion of the Enablement Rejection

The Office Action maintains the rejection of claims 3, 5-8, 12, 26, 67-69, 72, 87, and 88 under 35 USC 112, first paragraph, as allegedly lacking enablement. Specifically, the Office Action contends that one skilled in the art could not predictably produce and test each cancer peptide variant encompassed by the broad scope of the claims with a reasonable

expectation of success without undue experimentation. This rejection is traversed for the reasons set forth below.

The rejection as it pertains to variant peptides having at least 85% sequence identity to amino acids 127-136 of SEQ ID NO: 4 is moot in view of the amendments to claims 3 and 26.

The factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue" include:

- (A) The breadth of the claims;
- (B) The nature of the invention;
- (C) The state of the prior art;
- (D) The level of one of ordinary skill;
- (E) The level of predictability in the art;
- (F) The amount of direction provided by the inventor;
- (G) The existence of working examples; and
- (H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988)

In the instant case, the nature of the invention is an isolated cancer peptide consisting of amino acids 55-62 of SEQ ID NO: 4 or amino acids 127-136 of SEQ ID NO: 4, or a functionally equivalent variant having at least 85% sequence identity to amino acids 53-62 of SEQ ID NO: 4. The cancer peptide optionally has 1 to about 10 additional contiguous amino acids of SEQ ID NO: 4 at the N-terminus of the cancer peptide. Also, the cancer peptide of the functionally equivalent variant thereof stimulates cancer antigen specific T lymphocytes.

The breadth of the claims is limited to those peptides *consisting of* one of two specific portions of SEQ ID NO: 4, amino acids 55-62 or amino acids 127-136, or variants thereof. The variants must have at least 85% sequence identity to amino acids 53-62 of SEQ ID NO: 4. The peptide or variant thereof can additionally consist of up to 10 contiguous amino acids of SEQ ID NO: 4. Furthermore, the claimed peptides and variants are limited to those that stimulate cancer antigen specific T lymphocytes.

The level of one of ordinary skill, as evident by the prior art, is sufficient to make and use the instant invention. The art of making and testing peptides for antigenicity was well-known long before the filing date of the instant application (see Gill et al. *J Biol Chem* 242: 3308-3318 (1967), attached hereto). The art of testing peptides for the ability to stimulate antigen specific T lymphocytes was known at least as early as November 1994 (see Estaquier et al., *Eur J Immunol* 24(11):2789-95 (1994), abstract, attached hereto).

The originally-filed application provides an ample amount of direction provided by the inventor and several working examples, such that the level of predictability is reasonably high and the quantity of experimentation needed to make or use the invention based on the content of the disclosure is low. In particular, the specification at, for instance, page 31, lines 1-17, and page 44, line 1 through page 47, line 18, teaches a method of making and testing peptides and variants thereof for the ability to stimulate cancer antigen specific T lymphocytes. Over 20 specific peptides, including variants, were tested for the ability to stimulate T lymphocytes as measured by GM-CSF release. The results presented in Table 7 also provide teachings that one of ordinary skill in the art would take into consideration when making and testing other variants not explicitly set forth in Table 7. For instance, the results presented in Table 7 teaches that modification of a peptide at amino acid position 53 or 54 of SEQ ID NO: 4 with an aliphatic amino acid will yield a variant peptide that stimulates cancer antigen specific T lymphocytes. Table 7 also teaches that modification of a peptide at amino acid position 62 with a negatively-charged amino acid or a bulky aromatic amino acid will yield a variant peptide that does not stimulate cancer antigen specific T lymphocytes. The specification at page 10, lines 2-7, even provides a generic structural formula for a peptide variant that teaches how to make a peptide variant. Therefore, the originally-filed disclosure provides ample direction, including several working examples, such that one of ordinary skill in the art can predictably make a variant peptide without undue experimentation.

Therefore, in view of the foregoing, undue experimentation is not required of the ordinarily skilled artisan to make and use the instantly claimed invention. Accordingly, the originally-filed specification enables one of ordinary skill in the art to make and use the claimed invention. Therefore, Applicants request that the enablement rejection be withdrawn.

Discussion of the Anticipation Rejection

The Office Action maintains the rejection of claims 28, 29, and 83-85 under 35 USC 102 (a) as allegedly anticipated by Chen et al., *Proc Natl Acad Sci USA* 94: 1914-1918 (1997). This rejection is traversed for the reasons set forth below.

Claim 28 is directed to an immunogen comprising the composition of claim 26 alone or in combination with at least one immunostimulatory molecule, wherein the immunogen elicits a response by an antigen specific T lymphocyte. Claim 26 has been amended herein and is now directed to a composition comprising an isolated cancer peptide consisting of (a) amino acids 55-62 of SEQ ID NO: 4 or amino acids 127-136 of SEQ ID NO: 4, or a functionally equivalent variant thereof, wherein the functionally equivalent variant has at least 85% sequence identity with amino acids 53-62 of SEQ ID NO: 4, and (b) optionally 1 to

about 10 additional contiguous amino acids of SEQ ID NO: 4 at the N-terminus of the cancer peptide, wherein said cancer peptide or functionally equivalent variant stimulates cancer antigen specific T lymphocytes.

Therefore, even though claim 28 recites the transitional term "comprising," the claimed immunogen must comprise an isolated cancer peptide consisting of (a) amino acids 55-62 of SEQ ID NO: 4 or amino acids 127-136 of SEQ ID NO: 4, or a functionally equivalent variant thereof, wherein the functionally equivalent variant has at least 85% sequence identity with amino acids 53-62 of SEQ ID NO: 4, and (b) optionally 1 to about 10 additional contiguous amino acids of SEQ ID NO: 4 at the N-terminus of the cancer peptide, wherein said cancer peptide or functionally equivalent variant stimulates cancer antigen specific T lymphocytes.

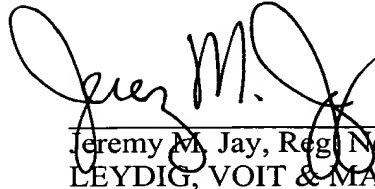
Chen et al. does not disclose a cancer peptide within the scope of claim 26. Namely, Chen et al. does not disclose an isolated cancer peptide *consisting of* (a) amino acids 55-62 of SEQ ID NO: 4 or amino acids 127-136 of SEQ ID NO: 4, or a functionally equivalent thereof, as defined by claim 26, and (b) optionally 1 to about 10 additional contiguous amino acids of SEQ ID NO: 4. Therefore, Chen et al. does not anticipate each and every limitation of claim 28.

In view of the foregoing, claim 28, and claims dependent thereon (claims 29 and 83-85) are not anticipated by Chen et al. Applicants, therefore, request that the anticipation rejection be withdrawn.

Conclusion

The application is considered in good and proper form for allowance, and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Jeremy M. Jay", is written over a horizontal line.

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Studies on Synthetic Polypeptide Antigens

XVIII. THE ROLE OF COMPOSITION, CHARGE, AND OPTICAL ISOMERISM IN THE IMMUNOGENICITY OF SYNTHETIC POLYPEPTIDES*

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SUMMARY

Studies of systematically varied series of synthetic polypeptides lead to several generalizations about the chemical factors involved in antigenicity. Polymers containing aromatic amino acids generally elicit more antibody than those not containing aromatic amino acids. This is not an exclusive property, however, since the incorporation of alanine can enhance immunogenicity in some cases. Tyrosine and phenylalanine are equally effective in enhancing antibody formation, but there is no correlation between the amount of antibody elicited and the amount of the aromatic amino acid in the polypeptide. The charge of the antigen plays essentially a restrictive role: completely charged and highly charged polypeptides are very poor immunogens.

The immunogenicity of D-amino acid polymers and the stereospecificity of the antibody response have been confirmed, and the reason for the difficulty in eliciting antibody formation has been established. Immunization with the relatively large doses of antigen used with L polymers (2 to 20 mg) does not generally elicit antibody formation with D antigens, but low doses (0.3 to 0.6 mg) of antigen administered over a long period of time induce antibody formation to a wide variety of D-polypeptides. The D polymers do not display a significant booster effect, and eventually the antibody response is abolished by continued injection of the antigen. Using this same low dosage schedule, polylysine and polyglutamic acid of both optical configurations also elicit antibody. Thus, the generalization can be made that small

doses of poor immunogens stimulate antibody formation and larger doses induce immunological paralysis.

The experiments described in this study suggest that the method needed to induce antibody formation is determined by quite general properties of the antigen (*e.g.* optical configuration, excessively high charge) and that the quantitative control of antibody production depends upon both the general properties and the detailed composition of the antigen (*e.g.* types of amino acids).

Synthetic polypeptide antigens¹ have been used extensively to define the chemical factors involved in immunogenicity and in the structure of antigenic sites; the results of these studies have been reviewed recently (3, 4). A wide variety of copolymers are immunogenic, and the amount of antibody elicited is consistently enhanced when tyrosine or phenylalanine is present (5-7). Simple, highly flexible copolymers of glutamic acid and lysine elicit antibody formation, so rigidity does not appear to be a requirement for immunogenicity. The over-all shape of the molecule is not a crucial factor, since linear, branched chain, and intramolecularly cross-linked synthetic polypeptides (8) are immunogenic. Molecular weight does not appear to be a factor, since an antigen as small as molecular weight 400 can elicit antibody formation (9). Charged homopolymers have not yet been shown to be antigenic (3-5), but the uncharged polymers polyproline (10, 11), multichain *N*-(3-hydroxypropyl)glutamic acid-

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† Junior Fellow of the Society of Fellows, Harvard University; the recipient of a Lederle Medical Faculty Award; and the recipient of a Research Career Development Award from the National Institutes of Health (K3-AM-5242), successively during the course of this study.

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¹ The nomenclature of the synthetic polypeptides is systematically defined in Gill (2). The superscripts refer to the molar percentages of each amino acid, and the number in parentheses is that of the preparation. The number of dinitrophenyl- residues per 100 amino acid residues is given in parentheses preceding the formula of the polymer and joined to it by a hyphen. The suffixes used for the modified residues in the polypeptides are: Ac, acetylated; Gu, guanidinated; D, deaminated; Me, methylated; and S, succinylated. D-Amino acid residues are designated by the prefix D; the absence of such a prefix indicates an L-amino acid residue.

TABLE I
Maximal amount of racemization in D-amino acid polymers

| Polymer | Mol. wt. | Degree of polymerization | D-Glu | D-Lys | D-Tyr | L-Amino acids ^a | | | | Residues | | | | | | L-Amino acids |
|---------------------------------------------------------------------------------|----------|--------------------------|-------|----------------|-------|----------------------------|------|-------|-------|----------|---|-----|----|-----|-----|---------------|
| | | | | | | Glu | Lys | Tyr | Total | Glu | | Lys | | Tyr | | |
| | | | | | | | | | | D | L | D | L | D | L | |
| | | | | | | | | | | | | | | | | |
| Poly D-Glu ⁵⁷ D-Lys ⁴⁴ | 46,000 | 295 | 57.2 | 42.8 | 0 | 0.58 | 3.62 | | 4.20 | 167 | 2 | 116 | 10 | | | 4.07 |
| Poly D-Glu ⁵⁵ D-Lys ³⁹ D-Tyr ⁶ | 93,000 | 590 | 54.5 | 39.1 | 6.4 | 0.37 | 2.0 | 0.052 | 2.42 | 320 | 2 | 220 | 11 | 37 | 0.3 | 2.25 |
| Poly D-Glu ⁵⁶ D-Lys ⁴⁰ D-Tyr ⁵ (No. 2)..... | 158,000 | 1,000 | 55.6 | 39.7 | 4.7 | 0.57 | 0.62 | 0.066 | 1.26 | 550 | 6 | 391 | 6 | 46 | 0.6 | 1.26 |
| Poly D-Glu ⁴⁸ D-Lys ³⁸ D-Tyr ¹⁴ | 170,000 | 1,080 | 48 | 38 | 14 | 0.50 | 0.49 | 0.18 | 1.17 | 513 | 6 | 405 | 5 | 149 | 2 | 1.20 |
| Poly D-Glu ⁴⁶ D-Ala ¹⁰ D-Tyr ¹⁰ | 120,000 | 940 | 59.6 | 0 ^c | 9.8 | 0.41 | | 0.09 | 0.50 | 557 | 3 | | | 91 | 0.7 | 0.39 |

^a From microbiological assay of the 12-hour hydrolysate (4 N HCl at 121°) corrected for racemization of the amino acids themselves (according to Shankman; see text).

^b From amino acid analysis.

^c D-Ala = 30.6%; assume no L-amino acid residues present.

tyrosine (12), and the hydroxypropylglutamide derivative of poly Glu⁶⁰Ala⁴⁰ (13) can elicit an immune response. There is, however, a correlation between the net charge of the antigen and that of the antibody (14). The response to a synthetic antigen varies in different species (3) and is genetically controlled within a species (15-17). The D-amino acid polymer poly D-Glu⁵⁵D-Lys³⁹D-Tyr⁶ elicits antibody formation under the usual conditions of immunization (18-22), whereas a variety of other D-amino acid polymers do not (23-27). Recent studies in the rabbit (22) and in the mouse (28, 29) have shown that the antibody response to the D-polypeptides is dose-dependent and that the amounts of polymer generally used for immunization probably induce immunological paralysis.

The present study was undertaken to investigate further the influence of composition, charge, and optical isomerism on the immunogenicity of synthetic polypeptides. The role of composition was explored by comparing synthetic polypeptides containing glutamic acid, lysine, and an aromatic amino acid with similar polypeptides having no aromatic amino acids. The influence of charge was investigated by measuring the amount of antibody elicited by synthetic polypeptides of systematically varied charge density. A wide variety of D-polypeptides were tested for their ability to elicit antibody formation when given by different immunization schedules, in order to confirm their immunogenicity, to explore further their antigenic behavior, and to show that the findings have broad validity for D-amino acid polypeptides and are not peculiarities of a few closely related polymers. Finally, based on the experience with the D-polypeptides, the immunogenicity of polylysine and polyglutamic acid of both optical configurations was re-examined.

METHODS

Synthetic Polypeptides—The polypeptides were synthesized by polymerization of the N-carboxyanhydrides in a 1% solution in benzene or in 95% benzene and 5% nitrobenzene with the use of sodium methoxide as the initiator at an anhydride to initiator ratio of 400. The polypeptides were purified and then characterized by a variety of hydrodynamic, spectroscopic, and chemical methods as described previously (5). In addition to the usual methods of characterization, all the D-polypeptides were examined by microbiological assay in order to determine the

amount of L-amino acid that each contained. These analyses were performed by S. Shankman, Los Angeles, California, by standard microbiological assay methods. The amount of racemization that occurred in the process of acid hydrolysis was determined by applying the same treatment with hot acid, for the same period of time, to D-glutamic acid, D-lysine, and D-tyrosine. The amount of L-amino acid in each polymer was corrected for racemization during hydrolysis. This value gives a maximum estimate of the amount of L-amino acid in a polymer prior to hydrolysis, since the probability of racemization in the hydrolysis of a polypeptide is greater than it is when the same treatment is applied to the amino acids themselves. The results of the microbiological assays are summarized in Table I.

Immunization—Male and female New Zealand white rabbits weighing 2.5 to 3 kg (3 to 4 months old) were used in all of the studies. The animals were all from the same group of breeders, hence, they were drawn from a relatively closed population.² One study was performed with *Cebus albafron* monkeys, and they were immunized as indicated in Table VIII.

A variety of adjuvants were tried in rabbits, and the most effective was Difco's complete Freund's adjuvant to which additional tubercle bacilli were added (3 mg per ml final concentration). Alum, alginate proteins, and pertussis organisms were also tried as adjuvants, but they did not show a consistent adjuvant effect with the synthetic polypeptides. Polymers dissolved in NaCl-phosphate buffer alone gave low and erratic antibody responses. Using complete Freund's adjuvant there was no difference in antibody response over a 10-fold dose range,

² Inbred rabbits show distinct variation in the amount of antibody elicited by a given antigen (16). Genetic variation is not a factor in these studies, however, since the rabbits were all drawn from the same heterogeneous population. Also, the different groups of rabbits that received poly Glu⁵⁵Lys⁴¹ or poly Glu⁵⁶Lys³⁹Tyr⁶ were immunized at various times throughout the course of the study, hence they serve as an "internal control" on the rabbit population: there were no differences in the antibody responses of these groups (Table III). In addition to the strains previously described (16), the San Juan strain of rabbits forms more antibody against poly Glu⁵⁶Lys³⁹Tyr⁶ than does the New Zealand white strain: 103, 323, 336, 443 µg of antibody nitrogen per ml in four San Juan rabbits, with the use of the usual immunization schedule as described under "Methods."

TABLE II
Effect of dose on antibody response to poly
Glu⁵⁵Lys³²Tyr¹⁵ (No. 3)

| Amount of antigen | | Time interval | Antibody response | | | Animals responding |
|----------------------|--------------------------------|---------------|-----------------------|------------------|------------------|--------------------|
| In Freund's adjuvant | In NaCl-phosphate ^a | | $\mu\text{g Ab N/ml}$ | $\pm\text{S.D.}$ | $\pm\text{S.E.}$ | |
| mg | mg | wks | | | | |
| 0.1 | 0.1 | 1 | 133 | 172 | 77 | 5/5 |
| 0.5 | 0.5 | 1 | 164 | 75 | 34 | 5/5 |
| 0.5 | 2.0 | 1 | 216 | 146 | 65 | 5/5 |
| 2.0 | 0.1 | 1 | 98 | 57 | 26 | 5/5 |
| 2.0 ^b | 2.0 | 1 | 120 | 103 | 52 | 4/4 |
| 10.0 | 10.0 | 2 | 135 | 58 | 15 | 15/15 |

^a Given subcutaneously 3 weeks later.

^b When pertussis organisms were used instead of Freund's adjuvant, the antibody response was $24 \pm 4 \mu\text{g}$ of antibody nitrogen per ml in five out of five rabbits.

e.g. when 0.2 to 20 mg of poly Glu⁵⁵Lys³²Tyr¹⁵ (No. 3) were used for immunization (Table II).³

The immunization schedule for all of the studies on the effect of composition and of charge on immunogenicity employed 10 mg of antigen in Freund's complete adjuvant followed in 3 weeks by 10 mg of antigen in NaCl-phosphate buffer; 2 weeks later the animals were bled. Different schedules for other studies are described in the specific contexts. In some of the immunochemical studies on the antibody to the D-polymers (Tables IX and X) a large amount of antibody was needed, so an aggregate of D-polypeptide with methylated BSA⁴ or with poly Lys⁷⁰Ala³⁰ incorporated into Freund's adjuvant was used for immunization; no antibody against the aggregating agent was detected (30).

The low dose-long term immunization schedule for the D-polymers had a primary course of 0.1 mg of antigen incorporated into Difco complete Freund's adjuvant (without additional tubercle bacilli) injected subcutaneously once monthly for 3 months; 1 week after the third injection, the rabbits were bled (Bleeding I). Three weeks after this bleeding, the animals were given 0.1 mg of antigen in Difco incomplete Freund's adjuvant and they were bled 1 week later (Bleeding II). This schedule was repeated for Bleedings III and IV.

Assay Methods—The precipitin reaction was performed as previously described (5). The sera were centrifuged at 12,000 rpm for 1 hour and then filtered through Whatman No. 42 paper. Equal volumes of serum and antigen (0.4 ml) were mixed and incubated at 4° for 5 days. The reaction mixtures were then centrifuged and the precipitates were washed twice with buffer and dissolved in 0.25 M acetic acid. The amount of antibody was determined from the absorbance of the solutions at 277 m μ with the use of a Beckman DU spectrophotometer equipped with microcells. This method allows the detection of 1 μg of antibody nitrogen per ml of serum. Sera from four animals immunized

³ The minimal tested dose of poly Glu⁵⁵Lys³²Tyr¹⁵ (No. 2) that gave an antibody response was 4 μg of antigen incorporated into Freund's complete adjuvant given monthly for 3 months. It elicited 0, 11, 15, 21, 49 μg of antibody nitrogen per ml in five New Zealand white rabbits which were bled 1 week after the last injection of antigen.

⁴ The abbreviations used are: BSA, bovine serum albumin; MeBSA, methylated bovine serum albumin; DNP-, dinitrophenyl-; DPE, dinitrophenylene.

according to the usual immunization schedule with Freund's adjuvant emulsified with NaCl-phosphate buffer were mixed with a variety of different synthetic polypeptide antigens to test for nonspecific precipitation. None was found; hence the materials in the Freund's adjuvant do not elicit antibody that reacts with any of the synthetic polypeptides.

The passive cutaneous anaphylaxis reaction was carried out as described by Ovary (31) and by Ben-Efraim, Fuchs, and Sela (32). A 0.1-ml portion of antiserum was injected intradermally into guinea pigs; one group was challenged 3 hours after injection and another group, 18 hours after injection. Various dilutions of the antiserum were used in each case, and the reaction was elicited in different groups of guinea pigs by intravenous injection of 0.05 to 5 mg of antigen mixed with Evan's blue. The reactions were read by killing the guinea pigs and examining the underside of the skin.

The complement fixation reactions were performed by the semimicro method of Wasserman and Levine (33).

RESULTS

Reproducibility—Two types of studies were undertaken to establish the biological and chemical reproducibility of the data; the same preparation of a polypeptide was used to immunize different groups of rabbits, and various preparations of

TABLE III
Antibody response to same polymer in different groups of rabbits

| Polymer | Antibody response | | | Animals responding |
|-------------------------------------------------------------------------------|-----------------------|------------------|------------------|--------------------|
| | $\mu\text{g Ab N/ml}$ | $\pm\text{S.D.}$ | $\pm\text{S.E.}$ | |
| Poly Glu ⁵⁵ Lys ³² | 51 | 28 | 8 | 12/12 |
| | 63 | 65 | 27 | 5/6 |
| | 31 | 46 | 14 | 9/11 |
| Poly Glu ⁵⁵ Lys ³² Tyr ¹⁵ | 106 | 42 | 13 | 10/10 |
| | 69 | 25 | 8 | 10/10 |
| | 104 | 100 | 38 | 7/7 |
| | 81 | 31 | 10 | 9/9 |
| Poly Glu ⁵⁵ Lys ³² Tyr ¹⁵ (No. 2) | 56 | 36 | 12 | 9/9 |
| | 59 | 31 | 13 | 6/6 |
| Poly Glu ⁵⁵ Lys ³² Tyr ¹⁵ | 126 | 73 | 26 | 8/8 |
| | 159 | 70 | 25 | 8/8 |
| Poly Glu ⁵⁵ Lys ³² Tyr ¹⁵ | 144 | 128 | 43 | 9/9 |
| | 162 | 90 | 40 | 5/5 |
| Poly Glu ⁵⁵ Lys ³² Tyr ¹⁵ | 143 | 77 | 26 | 9/9 |
| | 122 | 15 | 6 | 6/6 |
| Poly Glu ⁵⁵ Lys ³² Tyr ¹⁵ (No. 2) | 97 | 27 | 14 | 4/4 |
| | 87 | 54 | 19 | 8/8 |
| Poly Glu ⁵⁵ Lys ³² Tyr ¹⁵ (No. 4) | 149 | 130 | 58 | 5/5 |
| | 98 | 57 | 26 | 5/5 |
| DPE (2.0)-poly Glu ⁵⁵ Lys ³² Tyr ¹⁵ (No. 3D) | 89 | 85 | 30 | 8/8 |
| | 93 | 49 | 20 | 6/6 |
| Poly Glu ⁵⁵ Lys ³² Ala ³⁰ | 139 | 93 | 33 | 8/8 |
| | 84 | 54 | 18 | 9/9 |
| Poly Glu ⁵⁵ Ala ³⁰ Tyr ¹⁵ | 126 | 35 | 14 | 6/6 |
| | 266 | 117 | 39 | 9/9 |
| Poly Lys ⁷⁰ Ala ³⁰ | 349 | 87 | 36 | 6/6 |
| | 42 | 28 | 9 | 8/9 |
| Poly D-Glu ⁵⁷ D-Lys ⁴⁴ | 36 | 16 | 5 | 9/9 |
| | 0 | | | 0/13 |
| Poly D-Glu ⁵⁷ D-Lys ³² D-Tyr ¹⁵ | 0 | | | 0/11 |
| | 11 | 10 | 4 | 5/5 |
| | 15 | 14 | 6 | 6/6 |

the same polypeptide were used for immunization. The studies of the biological reproducibility of the antigens (Table III) show that the amount of antibody elicited by an antigen is statistically the same in different groups of rabbits. The studies on the chemical reproducibility show that different preparations of the same polypeptide elicit statistically the same amount of antibody (Table IV). The differences between groups of rabbits had to equal or exceed a probability value of 0.01 to be considered significant.

Role of Composition—Synthetic polypeptides of the glutamic acid-lysine series with or without aromatic amino acids were compared in order to test the effect of aromatic amino acids on the amount of antibody elicited. The results are summarized in Table V and they are plotted in Fig. 1. In general, the polypeptides containing aromatic amino acids elicit more antibody, especially those with a net charge density of +25 to +75, and phenylalanine and tyrosine are equally effective in enhancing antibody formation. There is no correlation between the amount of tyrosine or phenylalanine in a polymer and the amount of antibody elicited. The effect of the aromatic amino acids is consistent, but not unique, since poly Glu⁵⁵Lys²⁸Ala³⁰ elicits as much antibody as the aromatic amino acid-containing polymers. It is interesting in this connection that poly Glu⁵⁵Ala²⁸Tyr⁹ elicits more antibody ($p = 0.01$) than any of the glutamic acid-lysine-tyrosine polymers (Table V).

Role of Charge—The effect of the charge of the antigen on the amount of antibody elicited was examined with a series of glutamic acid-lysine and glutamic acid-lysine-tyrosine polymers. The data are summarized in Table V and they are graphed in Fig. 1. Completely charged homopolymers (poly Lys and poly Glu) or a modified copolymer (poly Glu⁵⁵Lys⁵⁴) do not elicit antibody formation with the immunization schedule used here. Highly charged polymers (e.g. poly Lys⁹⁰Tyr⁴) elicit only small amounts of antibody whether or not they contain aromatic amino acids. The best antigens fall in the range +75% to -75% net charge density and within this range there is no effect of charge on the amount of antibody elicited. An antigen with no net charge elicits a comparable amount of antibody, and this finding is consistent with the observation that uncharged polymers can be immunogenic (10-13). Thus, charge is not a requirement for immunogenicity, but excessively high charges can decrease or, under some conditions, abolish the ability to elicit antibody formation.

D-Amino Acid Polymers and Usual Immunization Schedule—All of the D-polypeptides studied are listed along with some of their properties in Table VI. The immunogenicity of poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁶ (18, 22) and that of a second preparation of the polymer, poly D-Glu⁵⁵D-Lys⁴⁰D-Tyr⁵ (No. 2), was confirmed.⁶ In addition, repeated injections of antigen were shown not to elicit a booster response, but rather to decrease the amount of

⁶ Rabbits immunized with poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁶ and poly D-Glu⁵⁵D-Lys⁴⁰D-Tyr⁵ (No. 2) (5 to 40 μ g of antibody nitrogen per ml) gave Type I Arthus skin reactions (\pm to $+$) (38), which were less intense and more erratic than those in rabbits immunized with poly Glu⁵⁵Lys²⁸Tyr⁹ and having a comparable amount of circulating antibody. An inbred strain of rabbits (strain C) which gave the highest antibody response to poly Glu⁵⁵Lys²⁸Tyr⁹ (16) did not form antibody when immunized with poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁶. Inbred strains of rats show genetic segregation in antibody formation to both the D and L polymers (S. J. Simonian, T. J. Gill, III, and S. N. Gershoff, *Fed. Proc.* **26**, 621 (1967)). Thus, there is genetic control of the antibody response to both the D and the L polymers.

TABLE IV
Antibody response to different preparations of same polymer

| Polymer | Preparation No. | Molecular weight | Antibody response | | | Animals responding |
|-----------------------------------------------------------------------|-----------------|------------------|-------------------|------------|------------|--------------------|
| | | | μ g Ab/N/ml | \pm S.D. | \pm S.E. | |
| Poly Glu ⁵⁵ Lys ⁴¹ | 1 | 101,000 | 36 | 41 | 7 | 36/39 |
| | 2 | 37,000 | 14 | 14 | 4 | 7/12 |
| | 3 | 152,000 | 35 | 33 | 11 | 9/9 |
| | 4 | 82,000 | 41 | 37 | 17 | 4/5 |
| Poly Glu ⁵⁵ Lys ⁴² Tyr ¹ | 1 | 81,000 | 67 | 115 | 33 | 11/12 |
| | 2 | 59,000 | 119 | 88 | 40 | 5/5 |
| Poly Glu ⁵⁵ Lys ³⁸ Tyr ⁶ | 1 | 110,000 | 91 | 52 | 8 | 38/38 |
| | 2 | 70,000 | 57 | 34 | 9 | 15/15 |
| Poly Glu ⁵¹ Lys ³² Tyr ¹⁶ | 2 | 33,000 | 100 | 49 | 15 | 11/11 |
| | 3 | 52,000 | 111 | 91 | 25 | 13/13 |
| | 4 | 86,000 | 98 | 57 | 26 | 5/5 |
| | 5 | 21,000 | 138 | 83 | 22 | 14/14 |
| DPE (2.0)-poly Glu ⁵¹ -Lys ³² Tyr ¹⁶ | 3D | 52,000 | 93 | 49 | 20 | 6/6 |
| DPE (1.5)-poly Glu ⁵² -Lys ³³ Tyr ¹⁵ | 4F | 86,000 | 104 | 26 | 13 | 4/4 |
| Poly Lys ⁷⁰ Ala ³⁰ | 1 | 564,000 | 34 | 23 | 5 | 17/18 |
| | 2 | 230,000 | 79 | 85 | 30 | 6/8 |
| Poly D-Glu ⁵⁵ D-Lys ³⁰ -D-Tyr ⁶ | 1 | 93,000 | 19 | 25 | 4 | 35/39 |
| | 2 | 155,000 | 9 | 10 | 2 | 17/22 |

antibody formed and finally to suppress the antibody response entirely. A similar course of immunization with L-polymers shows a marked booster effect (5). The immunization schedules and the antibody responses are summarized in Table VII. Poly D-Glu⁵⁵D-Lys³⁰, poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁴, poly D-Glu⁵⁵D-Ala³⁰D-Tyr¹⁰, poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁵ (No. 251), and poly D-Glu⁵⁵D-Ala³⁰D-Tyr⁸ (No. 236) did not elicit antibody with the usual immunization schedule. Since the coupling of DNP to poly-lysine was reported to render the derivative immunogenic (15), we tried to make poly D-Glu⁵⁵D-Lys³⁰ and poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁴ immunogenic by conjugating them with DNP. This maneuver did not render either polypeptide immunogenic, nor did it enhance the immunogenicity of poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁶ or poly D-Glu⁵⁵D-Lys⁴⁰D-Tyr⁵ (No. 2) (Table VII); the sera were assayed for antibody to the polypeptide and to the DNP-polypeptide conjugate.

The immunogenicity of poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁶ was shown also in *Cebus alba* monkeys (Table VIII). There is no booster response upon further injection of the antigen, and repeated injections of antigen abolish the antibody response.

Immunochemical Properties of Antibody to a Pair of Isomeric Polypeptides—The antibodies elicited by poly Glu⁵⁵Lys²⁸Tyr⁹ (No. 2) and by poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁶ have sedimentation constants of approximately 7 S. The antibody to the L-polymer precipitates gives a PCA reaction and fixes complement. The antibody to the D-polymer precipitates and fixes complement, but does not usually give a PCA reaction; two animals showed a slight reaction. On the other hand, the antisera elicited by poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁶ aggregated with methylated BSA give precipitin, PCA, and complement fixation reactions. The immunochemical properties of these antibodies are summarized in Table IX, and typical complement fixation curves are shown in Fig. 2.

The inability of the antisera to poly D-Glu⁵⁵D-Lys³⁰D-Tyr⁶

TABLE V
 Antibody response to synthetic polypeptides of different composition and charge

| Polymer | | Molecular weight ^a | Helix ^{a, b} | Average antibody response | | | Animals responding |
|-----------------|--------------------------------------------------------------|-------------------------------|-----------------------|-----------------------------|------------|------------|--------------------|
| Number | Formula | | | $\mu\text{g Ab N/ml}$ | $\pm S.D.$ | $\pm S.E.$ | |
| 17 | Poly Glu | 84,000 | 0 | 0 | | | 0/8 |
| 37 | Poly Glu ⁹⁷ Lys ³ | 125,000 | 0 | 0 | | | 0/9 |
| 18 | Poly Glu ⁹⁶ Tyr ⁴ | 114,000 | 0 | 5 | 5 | 2 | 5/8 |
| 21 | Poly Glu ⁷⁰ Ala ³⁰ | 61,000 | 0 | 7, 113 ^{c, d} | | | 2/8 |
| 19 | Poly Lys ⁷⁰ Ala ³⁰ | 564,000 | 10 | 34 | 23 | 5 | 17/18 |
| 20 | Poly Lys ⁹⁶ Tyr ⁴ | 487,000 | 0 | 13, 14, 18, 20 ^c | | | 4/10 |
| 16 | Poly Lys | 324,000 | 0 | 0 | | | 0/8 |
| 8 | Poly Glu ⁵⁹ Lys ⁴¹ | 101,000 | 20 | 36 | 41 | 7 | 36/39 |
| 23 | Poly Glu ⁴⁹ Lys ⁵¹ | 63,000 | 25 | 22 | 7 | 2 | 9/9 |
| 24 | Poly Glu ⁴² Lys ⁵⁸ | 72,000 | 35 | 50 | 65 | 15 | 10/17 |
| 22 | Poly Glu ⁴² Lys ⁵⁸ Ala ³⁰ | 74,000 | 40 | 97 | 50 | 13 | 15/15 |
| 28 | Poly Glu ⁸⁴ Lys ¹⁰ Tyr ⁶ | 110,000 | 0 | 56 | 73 | 20 | 13/14 |
| 32 | Poly Glu ⁷³ Lys ²⁷ Tyr ⁶ | 31,000 | 0 | 126 | 73 | 26 | 8/8 |
| 7 | Poly Glu ⁶⁸ Lys ³² Tyr ⁶ | 110,000 | 15 | 91 | 52 | 8 | 38/38 |
| 34 | Poly Glu ⁴⁷ Lys ⁵³ Tyr ⁶ | | 50 | 81 | 45 | 16 | 7/8 |
| 33 | Poly Glu ³⁷ Lys ⁶³ Tyr ⁶ | | 10 | 111 | 58 | 24 | 6/6 |
| 29 | Poly Glu ³⁶ Lys ⁶⁴ Tyr ⁶ | | 0 | 136 | 83 | 37 | 5/5 |
| 30 | Poly Glu ³³ Lys ⁶⁷ Tyr ⁶ | 300,000 | 0 | 150 | 112 | 30 | 14/14 |
| 27 | Poly Glu ¹¹ Lys ⁸⁹ Tyr ⁶ | 148,000 | 0 | 121 | 115 | 38 | 9/9 |
| 9 | Poly Glu ⁵⁶ Lys ⁴⁴ Tyr ⁶ | 81,000 | 20 | 67 | 115 | 33 | 11/12 |
| 13 | Poly Glu ⁶¹ Lys ³⁹ Tyr ¹⁶ | 31,000 | 5 | 135 | 58 | 15 | 15/15 |
| 11 | Poly Glu ⁵⁹ Lys ⁴⁰ Phe ¹ | 87,000 | 25 | 114 | 36 | 13 | 8/8 |
| 10 | Poly Glu ⁶² Lys ³⁸ Phe ⁵ | 63,000 | 15 | 101 | 46 | 15 | 9/9 |
| 12 | Poly Glu ⁵⁷ Lys ⁴³ Phe ⁹ | 61,000 | 15 | 65 | 80 | 28 | 7/8 |
| 38 | Poly Glu ⁶³ Ala ³⁷ Tyr ⁹ | 380,000 | 0 | 266 | 117 | 39 | 9/9 |
| 8S ^e | Poly Glu ⁵⁹ LysS ⁴¹ | | 0 | 0 | | | 0/8 |
| 8Ac | Poly Glu ⁵⁹ LysAc ⁴¹ | | 0 | 9, 24 ^c | | | 2/10 |
| 8Gu | Poly Glu ⁵⁹ LysGu ⁴¹ | | 35 | 9 | 14 | 5 | 4/8 |
| 8D | Poly Glu ⁵⁹ LysD ⁴¹ | | 5 | 43 | 5 | 2 | 8/8 |
| 8Me | Poly Glu ¹⁶ GluMe ⁴⁴ Lys ⁴¹ | | 65 | 28 | 10 | 4 | 8/8 |
| 24Me | Poly Glu ²⁸ GluMe ¹⁴ Lys ⁵⁸ | 75,000 | 40 | 22 | 8 | 3 | 8/8 |

^a See References 5, 34-37.

^b From the measurement of the b_0 parameter of the Moffitt equation in 0.11 M NaCl-0.04 M phosphate, pH 7.6.

^c Responses of individual animals.

^d This animal also gave a 4+ Arthus skin reaction.

^e The molecular weights of all the derivatives of poly Glu⁵⁹Lys⁴¹ are approximately 100,000 (37).

alone to elicit a PCA reaction requires comment. The antibody concentrations are high enough to give a reaction, since PCA reactions are elicited by antisera to the D-polymer aggregated with methylated BSA containing comparable amounts of antibody. In addition, the amounts of antibody in the sera are quite adequate for PCA reactions with a wide variety of other antigenic systems (31). Thus, it is probably not a question of the amount of antibody, but rather of a difference in the biological properties of the antibody. Only a minute fraction of the antibody elicited by poly D-Glu⁵⁶D-Lys³⁹D-Tyr⁶ alone may be able to give PCA reactions in guinea pigs, whereas most, or all, of the antibody elicited by the D-polymer aggregated with methylated BSA has that capability.

A series of isomeric polypeptides were examined for cross-reactivity in order to investigate the role of side chain residues in forming antigenic sites: any cross-reactions between enantiomorphs are due to side chain residues, since stereospecificity is confined to the polypeptide backbone. Previous studies (18, 23) failed to show cross-reactions, but the antibody concentra-

tions of these sera were relatively low. Therefore sera with large amounts of antibody were selected for the reinvestigation of cross-reactivity. The results of this study (Table X) show that there is a small degree of cross-reactivity between the D and L antigens, and that the amount of cross-reactivity follows a general pattern: as the tyrosine content of the cross-reacting antigen increases, the amount of antibody precipitated increases. This pattern holds true both for reactions within one enantiomorphic class and for reactions between enantiomorphs.

Joint Studies on Immunogenicity of D-Polypeptides—An attempt was made to reconcile the findings in our laboratory (18) and those in Professor Sela's (24) by jointly studying the method of immunization with the use of our antigen poly D-Glu⁵⁶D-Lys³⁹D-Tyr⁶ and his antigens poly D-Glu⁵⁹D-Lys⁴⁰D-Tyr⁶ (No. 251) and poly D-Glu⁴⁹D-Ala⁴⁰D-Tyr⁶ (No. 236). The immunization schedule of Fuchs and Sela (6) utilizes four intramuscular injections of 12.5 mg of polymer in complete Freund's adjuvant given at intervals of 10 days with a bleeding preceding each injection, and then 1 mg of antigen in NaCl-phosphate buffer given intra-

venously on alternate days for 4 days with a bleeding 1 week after the last injection. Subsequently, four other bleedings are taken at intervals of 3 weeks without any further injection of antigen. A total of 50 mg of antigen in Freund's complete adjuvant and 4 mg in NaCl-phosphate buffer is given. Using

this technique, no antibody was elicited by poly D-Glu⁵⁷D-Lys³⁹-D-Tyr⁶ in our laboratory nor by poly D-Glu⁶⁰D-Lys³⁴D-Tyr⁶ (No. 251) and poly D-Glu⁴⁹D-Ala⁴²D-Tyr⁶ (No. 236) in his laboratory. Thus, no antibody is elicited by any of the three D polymers by this immunization schedule. Our schedule is described under "Methods," and it employs 10 mg of antigen in Freund's complete adjuvant and 10 mg in NaCl-phosphate buffer. Using

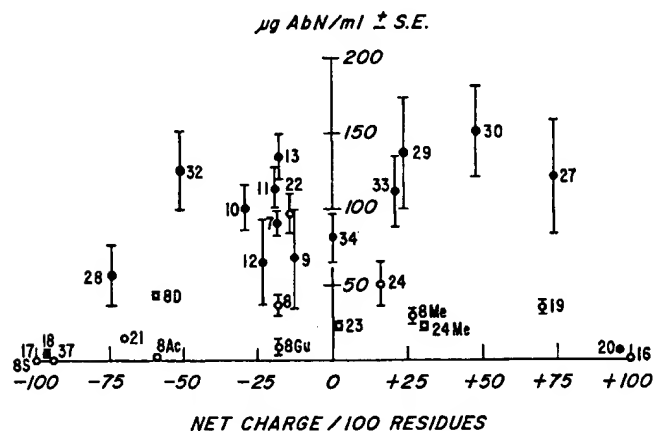


FIG. 1. The amount of antibody elicited by a systematically varied series of synthetic polypeptides containing glutamic acid and lysine (open circles) or glutamic acid, lysine, and tyrosine or phenylalanine (closed circles) plotted as a function of their charge density (net charge per 100 residues). The numbers refer to the polymers; the code and the properties of each polymer are summarized in Table V.

TABLE VI
Molecular weight and helical content of D-amino acid polymers

| Polypeptide | Molecular weight | Helical content ^a |
|----------------------------------------------------------------------------------------------|------------------|------------------------------|
| | | % |
| Poly D-Glu ⁵⁷ D-Lys ⁴³ | 46,000 | 15 |
| Poly D-Glu ⁵⁵ D-Lys ⁴⁴ D-Tyr ¹ | 82,000 | 25 |
| Poly D-Glu ⁵⁵ D-Lys ³⁹ D-Tyr ⁶ | 93,000 | 5 |
| Poly D-Glu ⁵⁵ D-Lys ⁴⁰ D-Tyr ⁶ (No. 2)..... | 155,000 | 15 |
| Poly D-Glu ⁴⁸ D-Lys ³⁸ D-Tyr ¹⁴ | 160,000 | 0 |
| Poly D-Glu ⁶⁰ D-Ala ³⁰ D-Tyr ¹⁰ | 120,000 | 0 |
| Poly D-Glu ⁶⁰ D-Lys ³⁴ D-Tyr ⁶ (No. 251) ^b | 44,000 | |
| Poly D-Glu ⁴⁹ D-Ala ⁴² D-Tyr ⁶ (No. 236) ^b | 33,800 | |
| Poly D-Lys ⁷⁰ D-Ala ³⁰ | 210,000 | |

^a Left-handed helix; helical content determined from the b_0 of the Moffitt equation obtained from measurements in 0.11 M NaCl-0.04 M phosphate, pH 7.6.

^b Kindly supplied by Professor Michael Sela, Weizmann Institute, Rehovoth, Israel (see Reference 24).

TABLE VII
Antibody response to several D-amino acid polypeptides and their DNP- derivatives in rabbits immunized according to usual schedules

| Polymer | Amount of antigen | | Time interval | Bleeding I | | Animals responding | Time interval | Amount of antigen | Time interval | Bleeding II | | Animals responding |
|---------------------------------------------------------------------------------|----------------------|--------------------------------|---------------|-----------------------|------------------|--------------------|---------------|-------------------|---------------|--------------------|----|--------------------|
| | In Freund's adjuvant | In NaCl-phosphate ^a | | $\mu\text{g Ab N/ml}$ | $\pm\text{S.D.}$ | | | | | | | |
| | mg | mg | | | | | | | | | | |
| Poly D-Glu ⁵⁷ D-Lys ⁴³ | 10 | 10 | 2 | 0 | | 0/24 ^b | 1 | 30 ^c | 1 | 0 | | 0/9 |
| (3 DNP)-poly D-Glu ⁵⁷ D-Lys ⁴³ | 10 | 2 | 1 | 0 | | 0/6 ^b | 1 | 2 | 1 | 0 | | 0/6 ^d |
| Poly D-Glu ⁵⁵ D-Lys ³⁹ D-Tyr ⁶ | 2 | 2 | 1 | 11 | 8 | 6/6 | 3 | 2 | 1 | 8, 11 ^e | | 2/6 |
| | 10 | 10 | 1 | 11 | 10 | 5/5 | | | | | | |
| | 10 ^f | 10 | 1 | 15 | 14 | 6/6 | | | | | | |
| | 10 | 10 | 2 | 33 | 40 | 7/11 | 1 | 30 ^c | 1 | 16 | 18 | 8/10 |
| | 1 ^g | | 1 | 19 | 16 | 12/12 | | | | | | |
| (5 DNP)-poly D-Glu ⁵⁵ D-Lys ³⁹ D-Tyr ⁶ | 10 | 2 | 1 | 6 | 5 | 4/6 | 0 | 2 | 1 | 3 | 2 | 3/6 ^h |
| Poly D-Glu ⁵⁵ D-Lys ⁴⁰ D-Tyr ⁶ (No. 2) | 2 | 2 | 1 | 8 | 10 | 3/6 | 2 | 2 | 1 | 1 ^d | | 1/5 |
| | 10 | 2 | 1 | 13 | 10 | 9/10 | 1 | 2 | 1 | 6 | 9 | 6/10 |
| | 10 | 10 | 1 | 3 | 3 | 5/6 | | | | | | |
| (3 DNP)-poly D-Glu ⁵⁵ D-Lys ⁴⁰ D-Tyr ⁶ (No. 2) | 10 | 2 | 1 | 9 | 11 | 3/5 | 0 | 2 | 1 | 10 | 10 | 3/5 ^h |
| Poly D-Glu ⁴⁸ D-Lys ³⁸ D-Tyr ¹⁴ | 10 | 2 | 1 | 0 | | 0/9 ^b | | | | | | |
| (11 DNP)-poly D-Glu ⁴⁸ D-Lys ³⁸ D-Tyr ¹⁴ | 10 | 2 | 1 | 0 | | 0/7 ^b | | | | | | |
| Poly D-Lys ⁶⁰ D-Ala ³⁰ D-Tyr ¹⁰ | 10 | 2 | 1 | 0 | | 0/8 | | | | | | |
| Poly D-Glu ⁶⁰ D-Lys ³⁴ D-Tyr ⁶ (No. 251) | 10 | 10 | 2 | 0 | | 0/6 | | | | | | |
| Poly D-Glu ⁴⁹ D-Ala ⁴² D-Tyr ⁶ (No. 236) | 10 | 10 | 2 | 0 | | 0/6 | | | | | | |

^a Given 3 weeks later; all subsequent injections of antigen were given in NaCl-phosphate buffer.

^b Fractionated and concentrated sera also showed the absence of antibody.

^c On alternate days 10 mg given intravenously.

^d An additional subcutaneous injection of 2 mg of antigen failed to elicit any antibody response.

^e Responses of individual animals.

^f The average antibody response 3 weeks later was $7 \pm 7 \mu\text{g}$ of antibody nitrogen per ml with five out of six animals responding.

^g Given as 0.35 mg once weekly for 3 weeks.

^h An additional subcutaneous injection of 2 mg of antigen did not significantly alter the antibody response.

ⁱ Antisera against (11 DNP)-poly Glu⁵⁵Lys³⁹Tyr¹⁴ (No. 3) contain $65 \pm 41 \mu\text{g}$ of antibody nitrogen per ml with eight out of eight rabbits responding.

TABLE VIII
Antibody response to poly D-Glu⁵⁵D-Lys³⁹D-Tyr⁶
in *Cebus albafron* monkeys

| Time | Bleeding | Amount of antigen | Antibody response ^a |
|------|----------|-------------------|-----------------------------------|
| wks | | mg | μg Ab N/ml |
| 0 | | 10 ^b | |
| 3 | I | 2 ^c | 27, 15, 15 |
| 5 | II | | 37, 39, 21 |
| 8 | III | 2 | 11, 16, 7 |
| 9 | IV | | 12, 21, 11 |
| 17 | V | 2 | —, ^d 5, 6 |
| 18 | VI | | —, ^d 4, 2 |
| 30 | VII | 2 | —, ^d 0, 0 ^e |
| 31 | VIII | | —, ^d 0, 0 ^e |

^a The individual antibody concentrations are arranged in the same order in each bleeding.

^b In Freund's adjuvant.

^c This and all subsequent injections of antigen were given subcutaneously in NaCl-phosphate buffer, pH 7.6, after the animals were bled.

^d This animal died.

^e Fractionated and concentrated sera also showed the absence of antibody.

TABLE IX
Immunochemical properties of antibody to poly Glu⁵⁵Lys³⁹Tyr⁶
(No. 2) and to poly D-Glu⁵⁵D-Lys³⁹D-Tyr⁶

| Antiserum | Serum No. | Anti-body nitrogen | Passive cutaneous anaphylaxis | Complement fixation titer |
|------------------------------------------------------------------------------------------|-----------|--------------------|-------------------------------|---------------------------|
| | | μg/ml | | |
| Poly Glu ⁵⁵ Lys ³⁹ Tyr ⁶ (No. 2) | 3 | 20 | ++++ | 320 |
| | 5 | 22 | ++++ | 80 |
| | 2 | 94 | ++++ | 640 |
| | 8 | 94 | ++++ | 1,280 |
| Poly D-Glu ⁵⁵ D-Lys ³⁹ D-Tyr ⁶ | Δ-7 | 11 | ± | 80 |
| | Δ-5 | 13 | 0 | 20 |
| | Δ-3 | 19 | 0 | 160 |
| | Δ-P1 | 19 | 0 | 160 |
| | Δ-90 | 19 | 0 | 80 |
| | Δ-P4 | 20 | 0 | 80 |
| | Δ-P3 | 35 | ± | 160 |
| | Δ-40 | 37 | +++ | 640 |
| Poly D-Glu ⁵⁵ D-Lys ³⁹ D-Tyr ⁶ aggregated with MeBSA | Δ-10 | 97 | ++++ | 2,560 |
| | Δ-20 | 104 | ++++ | 2,560 |
| | Δ-60 | 153 | ++++ | 5,120 |
| | Δ-30 | 155 | ++++ | 5,120 |
| | Δ-50 | 275 | ++++ | 10,240 |
| | Δ-10-f | 57 | +++ | 1,280 |
| Same, serum-fractionated | Δ-60-f | 104 | ++++ | 2,560 |
| | Δ-50-f | 218 | ++++ | 5,120 |

this method, we elicited antibody to poly D-Glu⁵⁵D-Lys³⁹D-Tyr⁶, but not to poly D-Glu⁵⁵D-Lys³⁹D-Tyr⁶ (No. 251) and poly D-Glu⁴⁹-D-Ala⁴⁹D-Tyr⁸ (No. 236). Thus, we confirm the original findings of the immunogenicity of our polymer and the lack of immunogenicity of the polymers of Sela when rabbits are immunized according to our schedule. According to Stupp and Sela⁶ our immunization schedule elicits antibody in two out of five rabbits

⁶ Y. Stupp and M. Sela, submitted for publication.

immunized with poly D-Glu⁵⁵D-Lys³⁹D-Tyr⁶ (11 and 13 μg of antibody nitrogen per ml, respectively); hence the immunogenicity of poly D-Glu⁵⁵D-Lys³⁹D-Tyr⁶ with the use of our immunization schedule is confirmed. The reason for the lack of immunogenicity of poly D-Glu⁵⁰D-Lys³⁴D-Tyr⁶ (No. 251) and poly D-Glu⁴⁹D-Ala⁴⁹D-Tyr⁸ (No. 236), which are structurally related to poly D-Glu⁵⁵D-Lys³⁹D-Tyr⁶ and cross-react with it, following immunization by either method, is not known; adequate amounts of these polymers were not available to test with the low dose-long term protocol (see below).

Low Doses of D-Polymer Administered over Prolonged Period of Time—Previous studies of the antibody response to the D-polypeptides and of their persistence in the tissues (20, 22) led to the postulate that their apparent lack of immunogenicity was due to immunological paralysis caused by the relatively large amounts of antigen generally used for immunization. To test this hypothesis, various groups of animals were immunized with seven D polymers and one L polymer according to the low dose-long term method given in "Methods." All of the D polymers elicited antibody formation (Table XI). Under these conditions of immunization neither the D nor the L polymers showed a significant booster effect.⁷ The antibody response to the L-polypeptide was the same as that elicited by a variety of other immunization schedules (Table II).

The addition of DNP- residues to poly D-Glu⁵⁷D-Lys⁴³ does not enhance the total amount of antibody it elicits (Table XI), so even with this immunization schedule DNP does not effectively enhance immunogenicity. Aggregation of the poor immunogen poly D-Glu⁵⁵D-Lys⁴⁴D-Tyr¹ with MeBSA elicits the same amount of antibody (Table XI) as would be expected from previous experiments with aggregates (30), and no booster effect is seen with continued immunization. Thus, the employment of this immunization schedule can overcome the barrier to antibody formation, but apparently it cannot effect quantitative changes in antibody formation.

Charged Homopolymers—In view of the demonstration of the immunogenicity of the D polymers, the ability of charged homopolymers to elicit antibody formation was reinvestigated with the use of the same schedule of immunization. They were shown to be immunogenic, although they were generally weak and erratic immunogens, especially the polyglutamic acids. The data are summarized in Table XII, and representative precipitin curves are shown in Fig. 3.

DISCUSSION

Aromatic amino acids consistently enhance antibody production, although they are not necessary to elicit it. Some antigens

⁷ When poly D-Glu⁴⁹D-Lys³⁹D-Tyr¹⁴ is aggregated with MeBSA and emulsified with Freund's adjuvant, there is a booster response demonstrable early in the course of immunization: 81, 64, 42, 30, 17 μg of antibody nitrogen per ml and a ± Arthus skin reaction (average) after two doses of 0.3 mg of aggregated polymer and 139, 169, 56, 44, 30 μg of antibody nitrogen per ml and a ++ Arthus skin reaction (average) following the third dose of antigen. In contrast to its enhancing behavior with poor antigens, aggregation with MeBSA does not enhance the amount of antibody elicited by a potent antigen. In fact, such aggregation can cause less antibody to be formed, but this difference is significant only at the $p = 0.05$ level: poly Glu⁵⁵Lys³⁹Tyr¹⁸ (No. 4) alone, 164 ± 75 (S.D.) μg of antibody nitrogen per ml and aggregated, 81 ± 24 μg of antibody nitrogen per ml (30); diphtheria toxoid alone, 379 ± 119 μg of antibody nitrogen per ml and aggregated, 241 ± 32 μg of antibody nitrogen per ml.

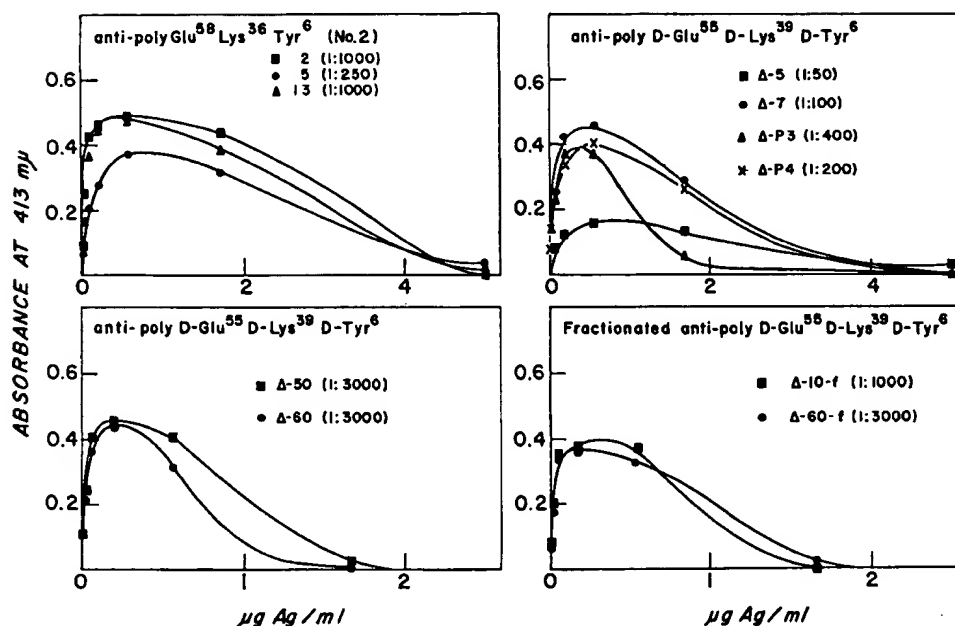


FIG. 2. Complement fixation curves for an enantiomorphous pair of synthetic polypeptides. The shapes of the curves are similar for both polymers. The fractionated serum is the globulin fraction obtained by precipitation with 18% Na_2SO_4 .

TABLE X
Serological cross-reactivity between L and D synthetic polypeptides^a

| Antigen | Antisera | | | | | |
|------------------------------------------------------------------------------|--------------------------------------------------|------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------------------|
| | Poly Glu ⁵⁸ Lys ³⁶ (No. 4) | Poly D-Glu ⁵⁸ D-Lys ³⁹ (No. 3) | Poly Glu ⁵⁸ Lys ³⁶ Tyr ⁶ (No. 2) | Poly D-Glu ⁵⁸ D-Lys ³⁹ D-Tyr ⁶ (No. 1) | Poly Glu ⁵⁸ Lys ³⁶ Tyr ⁶ (No. 4) | Poly D-Glu ⁵⁸ D-Lys ³⁹ D-Tyr ⁶ (No. 3) |
| | μg Ab N/ml | μg Ab N/ml | μg Ab N/ml | μg Ab N/ml | μg Ab N/ml | μg Ab N/ml |
| Poly Glu ⁵⁸ Lys ³⁶ (No. 4)..... | 49 | 2 | 494 | 5 | 244 | 4 |
| Poly D-Glu ⁵⁸ D-Lys ³⁹ | 4 | 66 | 18 | 101 | 14 | 111 |
| Poly Glu ⁵⁸ Lys ³⁶ Tyr ⁶ (No. 2)..... | 42 | 0 ^c | 666 | 11 | 376 | 7 |
| Poly D-Glu ⁵⁸ D-Lys ³⁹ D-Tyr ⁶ (No. 1)..... | 6 | 78 | 35 | 135 | 19 | 172 |
| Poly Glu ⁵⁸ Lys ³⁶ Tyr ⁶ (No. 4)..... | 45 | 9 | 649 | 18 | 386 | 15 |
| Poly D-Glu ⁵⁸ D-Lys ³⁹ D-Tyr ⁶ | 10 | 75 | 54 | 139 | 39 | 184 |

^a These studies were repeated with other sera, and the results were essentially the same as those presented here.

^b The antigens used to elicit antibody were aggregated with MeBSA or with poly Lys⁷⁰Ala³⁰ to increase their immunogenic potency. There was no detectable antibody to the aggregating agent (30).

^c Another serum showed 13% (9 out of 68 μg of antibody nitrogen per ml) cross-reactivity.

not containing aromatic amino acids can elicit comparable amounts of antibody, e.g. poly Glu⁵⁸Lys³⁶Ala³⁰, but the effect is not so consistent. Alanine is particularly potent in this regard, and its effect in enhancing immunogenicity when incorporated into synthetic polypeptides stands in marked contrast to its depression of immunogenicity when it is polymerized onto the lysine groups of gelatin (39-41) or ribonuclease (42). There is no correlation between the aromatic amino acid content of an antigen and the amount of antibody elicited; thus, it appears that the enhancement effect is a qualitative rather than a quantitative phenomenon. The enhancement of immunogenicity by aromatic amino acids is consistent with the results reported previously for synthetic polypeptides (5, 6), for nonimmunogenic polypeptides coupled with nucleosides (43) or with pyridoxal (44), for gelatin coupled with aromatic amino acids or with cyclohexylalanine (39-41), and for polylysine conjugated with DNP

(15).⁸ These materials enhance the ability of poor immunogens to elicit antibody, but only ferrocene (45) enhances the amount

⁸ The attachment of DNP residues to the potent antigen poly Glu⁵⁸Lys³⁶Tyr⁶ (No. 3) does not enhance the amount of antibody elicited: polypeptide alone, 111 ± 91 (S.D.) μg of antibody nitrogen per ml and DNP- derivative (11 residues per 100 amino acids), 65 ± 41 (S.D.) μg of antibody nitrogen per ml. In addition, the relatively weak antigen poly Glu⁵⁸Lys³⁶ (No. 3), which has 360 lysine residues, was conjugated with various amounts of DNP to test its effect on the amount and specificity of the antibody formed (usual immunization schedule). Conjugating up to 40% of the lysine residues with DNP did not alter the total amount of antibody elicited; the addition of more DNP gradually decreased the antibody response until all of the lysine residues were conjugated with DNP, at which point the polypeptide did not elicit any antibody. When 3% or less of the lysine residues were conjugated with DNP, more than 50% of the antibody was directed toward the DNP group; up to 40% conjugation, less than

TABLE XI

Antibody response to variety of *D*-amino acid polypeptides and typical *L*-polypeptide following immunization with multiple small amounts of antigen over prolonged period of time

| Polypeptide antigen | Bleeding I ^a | | Bleeding II | | Bleeding III | | Bleeding IV | |
|------------------------------------------------------------------------------------------------------------------|-------------------------------|---------|----------------------------------|---------|---------------------------------------|---------|--------------------------------|---------|
| | $\mu\text{g Ab N/ml}$ | Average | $\mu\text{g Ab N/ml}$ | Average | $\mu\text{g Ab N/ml}$ | Average | $\mu\text{g Ab N/ml}$ | Average |
| Poly <i>D</i> -Glu ⁵⁷ <i>D</i> -Lys ⁴³ | 34, 7, 7, 5, 4, 4 | 10 | 30, 12, 8, 15, 12, 6 | 14 | 21, 6, 7, 13, 4, 6 | 10 | 17, 0, 7, 12, 6, 6 | 8 |
| Poly <i>D</i> -Glu ⁵⁶ <i>D</i> -Lys ⁴⁴ <i>D</i> -Tyr ¹ | 54, 50, 18, 6, 6, 5 | 23 | 42, 42, 25, 10, 3, 9 | 22 | 30, 19, 13, 5, 4, 9 | 13 | 24, 12, 10, 5, 5, 7 | 10 |
| Poly <i>D</i> -Glu ⁵⁶ <i>D</i> -Lys ⁴⁹ <i>D</i> -Tyr ⁸ | 34, 23, 12, 10, 9, 0 | 15 | 34, 5, 15, 9, 13, 0 | 13 | 33, 4, 13, 4, 14, 0 | 11 | 25, 0, 9, 0, 6, 0 | 7 |
| Poly <i>D</i> -Glu ⁵⁶ <i>D</i> -Lys ⁴⁰ <i>D</i> -Tyr ⁵ (No. 2) | 40, 35, 13, 12, 11, 11 | 20 | 18, 21, 6, 47, 5, — ^b | 19 | 32, 16, 0, 33, 7, 7 | 16 | 8, 9, 0, 20, 0, 4 | 7 |
| Poly <i>D</i> -Glu ⁴⁸ <i>D</i> -Lys ³⁸ <i>D</i> -Tyr ¹⁴ | 27, 21, 6, 5, 0, 0 | 10 | 21, 7, 0, 8, 0, 0 | 6 | 20, 7, 0, 0, 0, 0 | 4 | 9, 7, 0, 0, 0, 0 | 3 |
| Poly <i>D</i> -Glu ⁴⁰ <i>D</i> -Ala ³⁰ <i>D</i> -Tyr ¹⁰ | 11, 10, 7, 0, 0, 0 | 5 | 12, 25, 4, 3, 0, 0 ^c | 7 | 10, 23, 3, 5, 0, 0 | 6 | 11, 7, 0, 2, 0, 0 ^d | 3 |
| Poly <i>D</i> -Lys ⁷⁰ <i>D</i> -Ala ³⁰ | 19, 12, 0, 0, 0, 0 | 5 | 14, 13, 0, 0, 0, 0 | 4 | 12, 8, 0, 0, 0, 0 | 3 | 0, 0, 0, 0, 0, 0 | 0 |
| (3 DNP)-poly <i>D</i> -Glu ⁵⁷ <i>D</i> -Lys ⁴³ | 49, 36, 13, 8, 6 | 22 | 23, 29, 10, 6, 4 | 14 | 13, 24, 9, 4, 4 | 11 | 8, 20, 8, 2, 2 | 8 |
| Poly <i>D</i> -Glu ⁵⁶ <i>D</i> -Lys ⁴⁴ <i>D</i> -Tyr ¹ aggregated with MeBSA | 131, 125, 108, 105, 68, 57 | 99 | 95, 105, 60, 98, 66, 38 | 77 | 90, 70, 69, 111, — ^b 51 | 78 | 86, 62, 57, 97, 87, 60 | 75 |
| Poly Glu ⁴³ Lys ³⁸ Tyr ¹⁵ (No. 3) | 296, 74, 56, 48, 45, 36 | 93 | 310, 68, 63, 55, 55, 55 | 101 | 267, 69, 70, 47, 47, 67 | 94 | 174, 69, 29, 40, 28, 41 | 64 |

^a The antibody concentrations in this and subsequent bleedings are arranged in the same order.

^b No blood could be obtained.

^c No significant change 4 weeks later (before the fifth injection of antigen): 12, 20, 0, 3, 2, 0, μg of antibody nitrogen per ml, respectively.

^d At 13 weeks after the last injection of 0.1 mg of antigen in incomplete Freund's adjuvant.

TABLE XII

Antibody response to *L*- and *D*-homopolymers following immunization with multiple small amounts of antigen over prolonged period of time

| Polypeptide antigen | Bleeding A ^{a,b} | Bleeding I | Bleeding II | Bleeding III | Bleeding IV |
|-------------------------|---------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| | $\mu\text{g Ab N/ml}$ | $\mu\text{g Ab N/ml}$ | $\mu\text{g Ab N/ml}$ | $\mu\text{g Ab N/ml}$ | $\mu\text{g Ab N/ml}$ |
| Poly Glu (No. 2)..... | 5, 3, 3, 3, 0, 0 | 2, 2, 2, 2, 0, 0 | 8, 8, 0, 11, 9, 6 | 2, 4, 0, 3, 0, 4 | 2, 4, 0, 2, 0, 4 |
| Poly <i>D</i> -Glu..... | 4, 3, 0, 0, 0, 0 | 3, 3, 0, 0, 0, 0 | 3, 3, 5, 0, 0, 0 | 3, 5, 5, 5, 4, 4 | 0, 0, 0, 0, 0, 0 |
| Poly Lys (No. 5)..... | 4, 4, 4, 3, 2, 0 | 8, 8, 4, 4, 7, 4 | 2, 5, 0, 14, 0, 0 | 0, 3, 0, 20, 0, 0 | 0, 2, 0, 20, 0, 0 |
| Poly <i>D</i> -Lys..... | 34, 19, 13, 8, 8, 7 | 20, 6, 6, 7, 5, 6 | 13, 12, 0, 8, 8, 6 | 6, 8, 0, 5, 6, 5 | 9, 8, 0, 0, 2, 0 |

^a Just before the third injection of antigen.

^b Antibody concentrations in the same order for each bleeding.

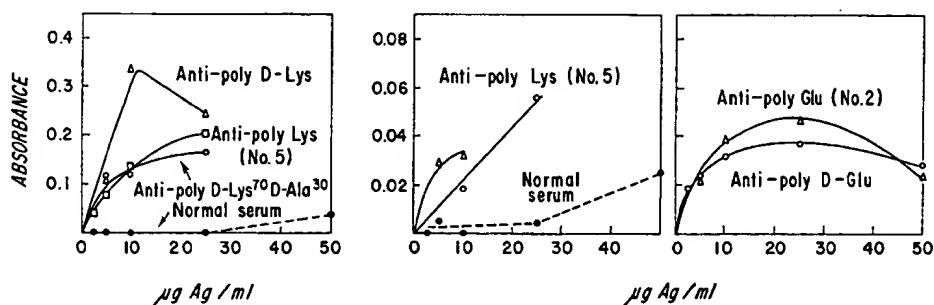


FIG. 3. Representative precipitin curves of the enantiomorphic homopolymers and of poly *D*-Lys⁷⁰*D*-Ala³⁰. The normal serum curves are representative of the amount of precipitate formed by mixing normal rabbit serum and poly *D*-Lys, poly Lys (No. 5), or poly *D*-Lys⁷⁰*D*-Ala³⁰; many other normal sera show comparable curves. Thus, the antisera show their precipitin curves in the region where there is negligible nonspecific precipitation due to the antigen alone.

of antibody elicited by both good and poor immunogens; the reason for this difference is not understood. Since aromatic

25% of the antibody was directed towards the DNP; and with greater amounts of DNP, less than 5% of the antibody was specific for the DNP group. Finally, conjugates containing up to 40% DNP-lysine residues cross-reacted completely with antisera to the parent polymer poly Glu⁴³Lys³⁸ (No. 3); with greater de-

amino acids are not necessary to elicit antibody formation, the role of rigidity in immunogenicity (39, 46) must be questioned, although it may still be operative in enhancing the amount of antibody produced. The charge of the antigen plays essentially

degrees of conjugation, the reactivity decreased rapidly and disappeared when 60% of the lysine residues contained DNP groups.

a restrictive role: completely charged and highly charged molecules are quite poor immunogens. There is no correlation between molecular weight or helical content and the amount of antibody elicited by the synthetic antigens, over the ranges studied (Tables IV and V).

There is a small amount of cross-reactivity between enantiomeric antigens, and it is greatest when both the cross-reacting antigen and the antigen used for immunization contain tyrosine. These observations show that side chain residues, especially that of tyrosine, can form a large enough portion of some antigenic sites to overcome the steric differences due to the polypeptide backbone. Similar evidence for the antigenic capability of the phenylalanyl side chain has been presented by Parker, Gott, and Johnson (47) in a study of an enantiomeric pair of tetrapeptide conjugates with the use of the fluorescence quenching method. Additional evidence indicating small amounts of cross-reactivity among isomeric antigens has been presented for synthetic polypeptides by Maurer (7) and for polytyrosyl gelatins by Sela and Fuchs (48).

The role of the tyrosine side chain as a determinant cannot explain the increased cross-reactivity of antisera to poly Glu⁵⁹-Lys⁴ and to poly D-Glu⁵⁹-D-Lys⁴ with polypeptides containing tyrosine, since the specificity of these antisera is towards glutamyl and lysyl residues. The increased cross-reactivity suggests that the tyrosine residues increase the hydrophobic character of the antigen and, therefore, the precipitability of their antigen-antibody complexes.

Synthetic polypeptides composed of D-amino acids are immunogenic, but less so than their L counterparts; they elicit antibody which is stereospecific for the D-amino acid polymer; and they can be degraded *in vivo* (20, 22, 28, 29). Stupp and Sela⁴ have shown that D-polypeptides must have at least 3% L-amino acids specifically incorporated in order to become immunogenic in rabbits under the usual conditions of immunization, in contrast to exclusively D-polypeptides. Since all but one of our D-polypeptides contained less than 3% L-amino acids (Table I), the amount of racemization is not biologically significant, and the D-amino acid polymers themselves are eliciting antibody formation. This conclusion is further supported by the stereospecificity of the antibody response, the low level of cross-reactivity between the D and L systems due to the side chains, and the rigid dose requirements for eliciting antibody with D-polymers in contrast to the broad dose ranges that are effective with L-polymers.

The usual doses of antigen (2 to 20 mg of antigen), which are relatively large, do not generally stimulate antibody formation with D-polypeptide antigens (Table VII). However, low doses of D-polypeptides over a prolonged period of time elicit antibody formation (Table XI). There is no significant booster effect with the D-polymers, and there is eventual suppression of the antibody response upon repeated injection of antigen. Similar findings have been reported when mice are immunized with D-polypeptides (28, 29). These observations indicate that the D-polypeptides behave immunologically like the pneumococcal polysaccharides in their low degree of immunogenicity, in the lack of a booster effect, and in the easy induction of paralysis. The paralysis is probably due to the prolonged tissue storage (20, 22) of the D polymers and their gradual release over a long period of time. Thus, very small amounts of antigen must be used over prolonged periods of time to induce antibody formation to D-amino acid polymers.

Immunization with small amounts of antigen over prolonged periods of time also elicited antibody formation to charged homopolymers. The previous inability to elicit antibody formation may be due to the induction of immunological paralysis by the amounts of antigen used, as in the case of most of the D-polypeptides.

The elicitation of antibody formation by D-polypeptides and by charged homopolymers indicates that all types of synthetic polypeptides are immunogenic in rabbits. However, there are important dosage restrictions which regulate their immunogenicity and compositional differences which regulate, in general, the magnitude of the antibody response within each enantiomeric class. There is a balance between stimulation and paralysis (49-53) for every antigen and this balance is under genetic control (3, 15, 17). Thus, we postulate that a poor immunogen induces paralysis easily, whereas a good immunogen stimulates an antibody response under a wide variety of conditions.⁹ Antigen metabolism may regulate antibody formation by controlling the amount of antigen left intact¹⁰ and capable of stimulating antibody formation over the critical period during which this event takes place. For example, poor immunogens such as D-polypeptides are poorly degraded (19-22), hence too much is present and paralysis ensues. Good antigens are rapidly degraded (20), so that over a wide range of doses, amounts optimal for stimulation only are present.

In conclusion, then, the chemistry of the antigen plays a crucial role in its ability to stimulate antibody formation and to provide specific antigenic sites. The structural basis of serological specificity is reasonably well understood, at least in principle, but the structural basis of induction and quantitative control of antibody formation is still only dimly perceived. The experiments described in this study suggest that the method needed to induce antibody formation is determined by quite general properties of the antigen (such as L or D isomer or excessively high charge) and that the quantitative control of antibody production depends upon both the general properties and the detailed composition of the antigen (such as the presence of aromatic amino acid). The biological orchestration of these chemical factors is then under careful genetic control.

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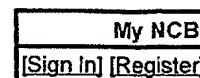
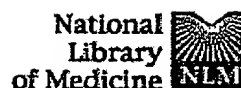
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⁹ Aggregation of an antigen with an oppositely charged macromolecule (30) may also act through a dosage effect. With poor immunogens the aggregate releases small amounts of antigen over a long time and they are adequate to induce antibody formation and to avoid paralysis. Aggregation of potent antigens does not affect the amount of antibody elicited (30) or may decrease it (Footnote 7), because it may release either an optimal or sub-optimal amount of antigen.

¹⁰ Following immunization with human serum albumin, Lapresle (54) and Lapresle and Webb (55) observed antibody to the intact molecule and antibody that reacted only with a splenic digest of the albumin. The latter antibody could have been elicited by some of the larger degradation products of the albumin formed *in vivo* after injection of the antigen.

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The mixotope: a combinatorial peptide library as a T cell and B cell immunogen.

Estaquier J, Gras-Masse H, Boutillon C, Ameisen JC, Capron A, Tartar A, Auriault C.

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We report a new approach in peptide vaccine strategy based on combinatorial synthesis. A library of 7.5×10^5 related peptides, termed mixotope, was derived from the sequence of the third hypervariable domain (V3 loop) of the human immunodeficiency virus (HIV) envelope protein. This preparation induced a strong immune response in all syngeneic and outbred rodents tested. The response directed against the mixotope included antibodies, CD4+ T helper cells (TH1 and TH2) and CD8+ T cells. In rodents immunized with the mixotope, the T cell response directed against individual V3 peptide sequences (BRU, MN, RF, SF2, and ELI) as measured by T cell proliferation and interleukin (IL)-2 production, was found to be major histocompatibility complex haplotype-dependent. However, additional experiments performed in mice indicated that selectivity was less restrictive when using IL-3 secretion to explore T cell activation. This combinatorial antigen could be considered as a series of agretopic motifs framing a multiplicity of closely related epitopes for T cell recognition and able to elicit a T cell and B cell repertoire. This new construct may therefore provide a basis for the design of future vaccine strategies.

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